The Annals of Clinical & Analytical Medicine
Original Research

Carbamazepine induces oxidative stress on rats' microvascular endothelial cells of the blood-brain barrier

Carbamazepine induces oxidative stress in the blood brain barrier

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Abstract

Aim: Carbamazepine (CBZ) is a commonly used anticonvulsant. Its effect on the blood-brain barrier(BBB) is poorly understood. Hence the current study had investigated the effect of CBZ on the isolated cultured microvascular epithelial cells of albino rats. Oxidative stress was investigated as the underlying mechanism of its cytotoxicity. Material and Method: Albino rats' microvascular endothelial cells (rMVECs) were isolated. CBZ induced cytotoxicity was evaluated by Alamar blue (AB) assay using concentrations range 1-1000μM. Oxidative stress markers as lipid peroxidation (TBARS), Catalase, superoxide dismutase(SOD), reduced glutathione(GSH), reactive oxygen species (ROS) were evaluated 24 hours after exposure to CBZ estimated IC50 and 50 μM. Also, the protective effect of anti-oxidation reduced GSH was studied. Results: AB assay showed that CBZ decreased the viability of the cells in relation to their concentrations and exposure durations. CBZ's 24 hours estimated IC50s was about 130μM. CBZ increased TBARS in the both 130 and 50 μM concentrations ($p = 0.0009$). CBZ significantly decreased SOD, catalase, and GSH levels in its estimated IC50s, while in the lower concentration (50 μM) only CBZ showed a significant effect on reduced GSH($p=0.003$). Also, CBZ increased significantly ($p=0.0018$) ROS with both tested concentrations. Addition of reduced GSH significantly decreased the cytotoxic effect of CBZ in its estimated IC50s ($p<0.0001$) and in the concentration of 50 μM ($p=0.0005$). Discussion: CBZ can be cytotoxic to the BBB cells with disruption of its integrity which may expose the nervous system to various harmful circulating molecules. Also, co-administration of antioxidants with CBZ may have a protective role.

Keywords

Carbamazepine; Blood-Brain Barrier; Microvascular Endothelial Cells; Anticonvulsant; Oxidative Stress

DOI: 10.4328/ACAM.6222 Received: 01.03.2019 Accepted: 27.03.2019 Published Online: 27.03.2019 Printed: 01.05.2020 Ann Clin Anal Med 2020;11(3):231-234
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Introduction

Carbamazepine (CBZ) is a worldwide widely used anticonvulsant drug which was firstly discovered in 1953 [1]. Its pharmacological action is primarily mediated by blockade of the voltage-gated sodium channels. It is mainly prescribed for the management of cases of epilepsy and neuropathic pain [2]. It is used also used in schizophrenia and bipolar disorder [3,4]. Its clinical use may be limited by its side effects which may be represented by skin rashes, bone marrow depression, and, suicidal ideation and confusion [5,6]. In cases of overdose, CBZ toxicity is mainly manifested by a wide range of clinical symptoms including blurred vision, drowsiness, slurred speech, ataxia, tremors, seizures, and bullous skin lesions [7].

The blood-brain barrier (BBB) means the unique properties of the microvessels of the central nervous system (CNS), which are non-fenestrated vessels with certain properties to regulate the movement of solutes between the blood and the CNS [8,9]. The main function of their barrier is to keep an optimal neuronal function, as well as CNS protection from pathogens and toxins [10]. BBB vessels are made of the endothelial cells lining of the vessels, which are forming the main part of the barrier. The interaction of these cells with other types of cells such as mural cells, immune cells, and glial cells are critical. [11].

CBZ is mainly targeting CNS for its therapeutic effect. Several studies had evaluated its transport efficacy through the BBB and its interaction with the other centrally acting drugs using different models [12-14]. In addition, it is postulated that the resistance to the therapeutic effect of anticonvulsant is mainly caused by the BBB efflux transporter P-glycoprotein (P-gP) which reduce the active part of the drug which reaches the CNS and induces the targeted therapeutic effect and studies have shown higher levels of P-gP transporter expression among epileptic pharmaco-resistant patients [15-17].

CBZ was shown to be cytotoxic and genotoxic for different cell line and living models [18-21]. Oxidative stress was shown to play a role in CBZ induced side effects and cytotoxicities using different models, concentrations, and exposure durations with significant inhibition of antioxidant enzymes' activities [20, 21]. However, the effect of CBZ on the BBB cells is poorly understood. Hence the current study had investigated the effect of CBZ on the isolated cultured microvascular epithelial cells of albino rats using a wide range of concentrations. Oxidative stress was investigated as the underlying mechanism of its cytotoxic effect. In addition, the protective effect of the reduced glutathione (GSH) was evaluated.

Material and Methods

Chemicals and reagents; In this study, CBZ and all reagents were purchased from Sigma (St. Louis, MO, USA), unless another source is specified. CBZ was dissolved in DMSO. Collagenase/ dispase and basic fibroblast growth factor (bFGF) were obtained from Roche Molecular Biochemicals (Indianapolis, IN, USA). Ham's F-10 nutrient mixture Fetal bovine serum and Horse serum were obtained from Gibco BRL (Grand Island, NY, USA). Modified Hanks was prepared following Daunt et al. (2005) [22]

Animal and treatment; Albino rats were used. All experiments were conducted after approval from the Mansoura Faculty of Medicine ethical committee. Rats were sacrificed under sodium pentobarbital anesthesia.

Isolation of rat cerebral microvascular endothelial cells (rCMECs); Isolation of rCMECs was based on a modified protocol as described before [23]. Finally, cells were purified by 33% continuous Percoll gradient. Cells were collected and washed and plated on 35 mm collagen IV/fibronectin-coated plates (both 0.1 mg/ ml). Isolated cells were maintained in the Endothelial Cell Medium supplemented with 4µg/ml puromycin and 100mg/ml heparin [24]. After 3 days, puromycin was removed from the media. Isolated cells were characterized by the excess expression of proteins of the ATP-binding cassette transporters P-gP and breast cancer resistant proteins were characterized by western blotting.

Alamar blue (AB) assay; AB assay was used as an indicator for cell viability. Following Elmorsy et al. (2015) [25], cells were seeded at $1x10^4$ cells per well in 96-well plastic plates and incubated till confluence. Then, cells were incubated for a 3, 6, 12, 24 and 48 hours in the presence of the CBZ in concentrations (0.1, 1, 10, 100, and 1000 µM respectively) or its vehicles. The AB absorbance values were expressed as a percent of the vehicle control (defined as 100%). Each drug concentration was tested in 3 wells in each experiment and experiments were performed in triplicates.

Measurements of oxidative stress markers; Cells were treated with CBZ (130 AND 50 µm) for 24 hours. Thiobarbituric acid reactive substances (TBARS) were quantified as markers of lipid peroxidation [26]. TBARS assay performed according to Alam et al. (2013) [27]. Superoxide dismutase (SOD) activity measurements were based on SOD-mediated inhibition of the reduction of nitroblue tetrazolium to blue formazan by superoxide anions as described by Beauchamp and Fridovich (1971) [28]. Units of SOD activity were expressed in terms of mg of total protein. While catalase (CAT) activity was assayed colorimetrically at 620 nm and expressed as µmoles H2O2 consumed/min/mg of protein using the method described by Sinha et al. (2008) [29]. Reduced GSH was determined based upon the original method of Ellman as described by Ullah et al. (2011) [30].

ROS detection; 3,7-dichlorodihydrofluorescein diacetate (DCF-DA) assay was used to detect the presence of reactive oxygen species (ROS), 24h post-treatment. Cells were treated with CBZ in both IC50 (130µm) and 50µM concentrations for 24 hours. The assay was done following Elmorsy et al. (2014) [31]. Antimycin A (10mM for 30 min) was used as the positive control, while wells with non-stained cells were used as the blank. The experiment was performed in triplicate, with triplicate of each treatment concentrations per experiment.

Effect of Reduced GSH; AB assays were repeated in the presence of 10µM reduced GSH. AB and comet assays were performed as shown in the previous sections.

All statistical procedures were performed using PRISM 5 (GraphPad Software Inc., San Diego, CA). IC50s were estimated by log concentration versus variable response using the best fit value. For comparisons, one-way ANOVA with Dunnet's post-test was used. Statistical significance is defined as $P<0.05$.

Results

This work was conducted to study the cytotoxic effect of CBZ on isolated rMVECs. AB assay showed that CBZ decreased the viability of the cells in relation to their concentrations and exposure durations (Figure 1). CBZ estimated IC50s are shown in Table 1.

The oxidative stress triggered by CBZ increased TBARS in the both estimated IC50s and 50 µM concentrations (p = 0.0009) (Figure 2A). The activity of the cellular redox regulating enzymes, CAT, and SOD, and the cellular levels of reduced GSH have been investigated. CBZ significantly decreased SOD, CAT,

and GSH levels in its estimated IC50s, (Figures 2B-D) while in the lower concentration (50 µM) only CBZ showed a significant effect on reduced GSH(p=0.003) with an insignificant effect on both SOD and CAT (Figures 2B-D).

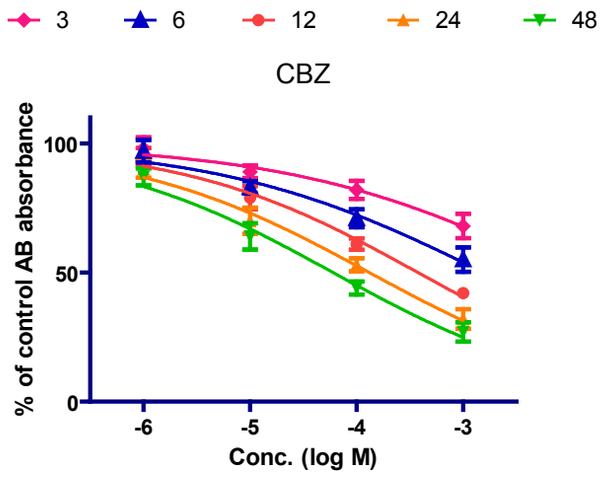

Figure 1. Cytotoxic effect of carbamazepine (CBZ) on the isolated rats' cerebral microvascular endothelial cells (rCMECs). Data were shown as means ± SD.

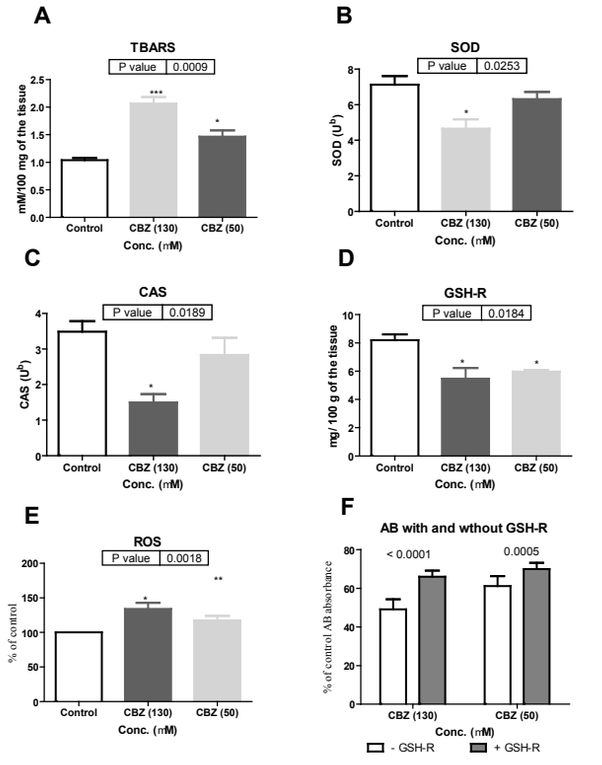

Figure 2. The effect of carbamazepine (CBZ) (130 and 50 µM) on the redox state of the isolated rats› microvascular endothelial cells of the blood-brain barrier. Oxidative stress biomarkers include; lipid peroxidation marker, TBARS (2A), superoxide dismutase (SOD) (2B), catalase (CAT) (2C), reduced GSH (GSH-R) (2D), and reactive species production (ROS) (2E). Reduced GSH significantly reduced their cytotoxic effect (2F). Data were expressed as mean±SD. The significance is indicated in the figures as *** for p<0.001, **for p<0.01 and *for p<0.05.

DCFDA assay showed significantly increased production of ROS (0.0002 ANOVA) with both CBZ in both tested concentrations (figure 2E). Addition of reduced GSH significantly decreased the cytotoxic effect of CBZ in its estimated IC50s (p<0.0001 for each) and in the concentration of 50 µM (p=0.0005) (Figure 2E).

Discussion

This work was conducted to study the cytotoxic effect of CBZ on isolated rMVECs. AB assay showed that CBZ decreased the viability of the cells in relation to their concentrations and exposure durations. The oxidative stress triggered by CBZ significantly increased TBARS in the treated cells. Oxidative stress studies showed that CBZ significantly decreased SOD, CAT, and GSH levels in its estimated IC50s while in the lower concentration (50 µM) CBZ showed a significant effect on reduced GSH. DCFDA assay showed significantly increased the production of ROS in CBZ treated cells. Addition of reduced GSH significantly decreased the cytotoxic effect of CBZ and CBZ-E on the isolated rMVECs.

The cytotoxic effect of CBZ was investigated using a wide range of concentrations from 1-1000 µM which cover all therapeutic, supra-therapeutic and toxic levels. The therapeutic level of CBZ was reported to be up to 12 mg/L (around 50µM), while the concentration of CBZ was reported to around 170 µM in cases of significant CBZ overdoses [32,33,34]. Higher concentrations were used to allow testing the chronic effect of the drug within the limited time frame of the experiment.

CBZ was reported to be cytotoxic to rMVECs in a concentration and time-dependent manner. Other studies had shown that CBZ was cytotoxic to other cell lines as Laville et al. (2003) who reported that CBZ was cytotoxic to rainbow trout hepatocytes (PRTH) and PLHC-1 fish cell line with estimated IC50s 318 and 650 µM respectively, which are higher than our estimated IC50s [19]. This may be due to lower levels of cytochrome enzymes in the endothelial cells in comparison to the hepatocytes cell lines as it was proved that higher level of cytochrome enzymes can enhance CBZ metabolism and lower its cytotoxic levels in the human embryonic kidney (HEK) cell line [35]. Furthermore, overexpression of cytochrome P450 was reported in patients with drug-resistant epilepsy due to reduced CBZ bioavailability [36].

The oxidative stress triggered by CBZ significantly increased TBARS in the treated cells and significantly decreased SOD, CAT, and GSH levels with a significant increase in the production of ROS in CBZ treated cells. This is in line with the previous studies as Li et al. (2010) who reported that CBZ in concentrations 2-20mg/L can induce oxidative stress in carp spermatozoa with significant inhibition of superoxide dismutase (SOD) and GSH peroxidase within 2 hours post-exposure [20]. CBZ was also shown to induce oxidative stress in rainbow trout brain tissues with long term exposure with sublethal concentrations of CBZ (1.0 µg/L, 0.2 mg/L or 2.0 mg/L) for up to 42 days [21]. In a human study, it was observed that decreased levels of total antioxidant capacity with increased total oxidative stress indices among epileptics maintained on CBZ in comparison to the other healthy group [37]. Antioxidant reduced GSH was shown to have a protective effect with better endothelial cells viability

Table 1. The IC50s, estimated by alamar blue for carbamazepine (CBZ) cytotoxicity on the isolated rats' microvascular endothelial cells of blood brain barrier. IC50s were estimated from the best fit of data with 95% confidence intervals (upper and lower limits).

	3h			6h			12h			24h			48h		
	M	UL	LL	M	UL	LL	M	UL	LL	M	UL	LL	M	UL	LL
CBZ	9060	4760	17240	1572	1083	2281	376.5	308.8	459.2	131.5	108.1	160	60.2	49.51	73.2

when co-treated with GSH.

The findings of the current study are important as they show that CBZ may be cytotoxic to the BBB endothelial cells. Hence it may disrupt the membrane integrity which may expose the CNS to various harmful circulating pathogens and molecules. This also may increase the CNS levels of other drugs and may be a source of drugs interaction with CBZ. In addition, our findings support the co-administration of antioxidants with CBZ, as it may protect the endothelial cells and decrease the toxic and side effects of CBZ.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

Funding: None

Conflict of interest

None of the authors received any type of financial support that could be considered potential conflict of interest regarding the manuscript or its submission.

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How to cite this article:

Elmorsy EM. Carbamazepine induces oxidative stress on rats' microvascular endothelial cells of the blood-brain barrier. Ann Clin Anal Med 2020;11(3):231-234